ORIGINAL ARTICLE

Optical delivery of multiple opsin-encoding genes leads to targeted expression and white-light activation

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In photodegenerative diseases such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD), progressive loss of vision occurs as a result of degeneration of the periphery of the retina and the macula, respectively. Current optogenetic stimulation-based approaches to vision restoration offer the advantages of cellular specificity, high resolution, and minimal invasiveness over electrode arrays; however, the clinical translation of optogenetic activation suffers from the lack of a method for the delivery of opsins into spatially targeted regions of a retina that has degenerated. Non-targeted opsin delivery through viral or non-viral methods to non-photodegenerated retinal areas will perturb these already functioning retinal regions. Furthermore, viral methods are subject to limitations on the delivery of large plasmids, such as fusion constructs of multiple spectrally separated opsins (e.g., channelrhodopsin-2 (ChR2), chimeric opsin variants (C1V1), ReaChR), which can provide higher photo-excitability than can a single narrow-band opsin under ambient light conditions. Here, we report the ultrafast near-infrared laser-based spatially targeted transfection of single and multiple opsins and present a comparison with the opsin expression distribution achieved using another non-viral, but non-targeted, transfection method, lipofection. Functional evaluation of cells transfected with multiple opsins using the laser method revealed a significantly higher white-light-induced photocurrent than in cells expressing a single opsin (ChR2). The laser-assisted targeted delivery of multiple opsin-encoding genes to the peripheral retina/macula is ideal for sensitizing retinal areas that have degenerated, thus paving the way toward the restoration of lost vision in RP/AMD patients.

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INTRODUCTION

The current methods for the optogenetic restoration of vision include the use of either channelrhodopsin-2 (ChR2) for non-specific stimulation of the retina¹ or promoter-specific approaches such as those using Thy1 for targeting retinal ganglion cells (RGCs)^{2–4} and mGluR6 for targeting ON bipolar cells⁵. Furthermore, the expression of chloride-channel opsin (halorhodopsin, NpHR⁶) in longer-persisting cone photoreceptors has shown promise for vision restoration⁶. The optogenetic activation method is very promising because it requires only a low intensity of light, which can be delivered by a light-emitting diode or laser^{7–10}. In addition, optogenetic approaches offer several advantages over electrical stimulation, such as cellular specificity, high temporal and spatial resolution, and minimal invasiveness^{7–8,11–14}. However, the clinical translation of optogenetic activation for vision restoration suffers from the lack of a method for the delivery of opsin-encoding genes into spatially targeted regions of a retina that has degenerated (e.g., the periphery of the retina in retinitis pigmentosa (RP) or the macula in age-related macular degeneration (AMD)). Non-targeted opsin delivery through viral or non-viral methods to residual (non-photodegenerated) retinal areas will cause complications in the functioning of these areas that have not degenerated by interfering

with the light-evoked potential in multiple layers of the retinal circuitry.

Recently, there has been considerable interest in the optical transfection of cells using ultrafast pulsed light^{15–23} because this approach offers selective targeting and higher efficiency and viability (>90% reported *in vitro*²²) than other methods. Furthermore, femtosecond (fs) near-infrared (NIR) laser-based transfection has been shown to be safe, resulting in high efficiency and high survival rates (93%) of optopored embryos during development²⁰, as well as suitable for *in vivo* gene delivery²⁴. We have demonstrated²⁵ the use of an ultrafast NIR laser microbeam for the optical delivery of opsin (ChR2)-yellow fluorescent protein (YFP) plasmids to spatially localized regions of the retina. Because non-linear interaction between the ultrafast laser beam and the cells occurs only within 1 μm of the focus, only the ganglion cell layer (which is proximal to the laser beam) or even only a single RGC can be sensitized to light. However, to achieve higher cell sensitivity to ambient broadband (white) light, it is advantageous to deliver multiple, spectrally separated opsins into the targeted cells. The delivery of large fusion constructs of multiple spectrally separated opsins (e.g., ChR2, chimeric opsin variants (C1V1), ReaChR) is challenging for the currently achievable safe viral methods. The sepa-

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rate delivery of different opsins by multiple viral vectors can lead to immune reactions and may cause unwanted transfection of non-targeted tissue. Although several non-viral approaches, such as electroporation and lipofection, exist, these methods suffer from low transfection efficiency, loss of cell viability, and non-targeted transfection, similar to viral methods. Ultrafast NIR laser-mediated optoporation^{23,25} can address these issues and holds promise for the delivery of large multi-opsin constructs via targeted transfection with single-cell resolution and high efficiency.

Herein, we report a comparative study of lipofection vs. the laser transfection of both single and multiple opsins. The fs laser transfection method was found to be capable of delivering large (>10 kb) multi-opsin constructs. The membrane-to-intracellular expression ratio was found to be higher in the case of laser transfection than lipofection because of less perturbation of cellular morphology. A higher photocurrent was observed in the case of HEK293 cells transfected with multiple opsins than in ChR2-sensitized HEK293 cells under white-light stimulation. The application of the spatially targeted laser transfection of multiple opsins in retinas that have degenerated will allow stimulation of the RGCs by broadband white light, leading to vision restoration in RP and AMD patients.

MATERIALS AND METHODS

Opsin-encoding plasmid preparation

The opsin-encoding plasmids were eluted with nuclease-free water upon receipt and stored at -20°C . For transformation, 1 μL of the DNA (10 ng) was added to a vial of JM109 (competent *Escherichia coli*) cells and then was mixed gently. The mixture-containing vials were incubated on ice for 30 min, and the cells were subjected to heat shock for 60 s at 42°C without shaking. The vials were then removed from the 42°C bath and placed on ice for 2 min, and 250 μL of S.O.C. medium was added to each vial. The vials were tightly capped and shaken horizontally at 37°C for 1 h at 220 rpm in a shaking incubator. Then, 100 μL of the transformation mixture was uniformly spread using a lazy-L spreader on a pre-warmed Luria-Bertani (LB) agar plate (Amp⁺). The plates were incubated at 37°C overnight, after which visible colonies appeared. A single colony of transformed bacteria was inoculated and grown overnight in 2 mL of pre-warmed LB medium in a shaking incubator at 220 rpm. To obtain the highest possible plasmid concentration, four individual colonies were chosen. Commercially available PureYieldTM Plasmid Miniprep/Maxiprep kits (Promega, Madison, WI, USA) were used to isolate and amplify the plasmids.

White-opsin synthesis

MultiSite Gateway[®] Technology²⁶ was used to fuse multiple opsin-encoding genes (ChR2, C1V1, ReaChR) to create White-opsin (Supplementary Fig. S1). In the 4-fragment recombination reaction, four PCR products (promoter, ChR2, C1V1, and ReaChR) flanked by specific attB or attBr sites and four donor vectors were used in separate BP recombination reactions to generate four entry clones. These four entry clones and the destination vector, pDESTTM R4-R3 Vector II, were used together in an LR recombination reaction to create the White-opsin construct.

DNA gel electrophoresis

To verify the opsin-plasmid constructs, DNA gel electrophoresis of the plasmids (with restriction enzymes) and a known DNA ladder (as a reference) was performed. First, 1 g of agarose was weighed and mixed with 100 mL of $1\times$ Tris-acetate-EDTA (TAE) buffer. The solution was

placed in a microwave for 1–3 min until the agarose was completely dissolved. The agarose solution was allowed to cool for 5 min. Ethidium bromide (EtBr) was then added to a final concentration of approximately $0.2\text{ }\mu\text{g mL}^{-1}$. The agarose gel was poured into a gel tray with a well comb in place. It was allowed to sit at room temperature until the gel solidified, and the agarose gel was then loaded into the gel box (electrophoresis unit). The gel box was filled with $1\times$ TAE until the gel was immersed. Restriction enzymes (Fermentas, MA, USA) were used to digest the plasmids. The DNA ladder (Promega) and the opsin plasmids (ChR2, ReaChR and White-opsin) were loaded into different lanes, and the gel was run at 100 V until the dye line was approximately 75% of the way down the gel. The power supply was then switched off, and the gel was carefully removed and placed under UV light for imaging (Gel Logic 212 PRO). The sizes of the bands (reference: DNA ladder) were found to match the expected sizes (Supplementary Fig. S1d). After the verification of proper band size, 5 L of bacteria were cultured to produce a large volume of plasmids using the PureYield Maxiprep kit (Promega). The concentration of the amplified plasmids was measured using a spectrophotometer (ND-1000, Nano Drop), and the results are shown in Supplementary Fig. S1e–S1g.

Cell culture

HEK293 cells were transfected with ChR2, ReaChR or the White-opsin construct using Lipofectamine (Life Technologies, Grand Island, NY, USA). 24 h after transfection, the HEK293 cells were cultured in Petri dishes and maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 with 10% fetal bovine serum, 0.2 mg mL^{-1} streptomycin, and 200 U mL^{-1} penicillin. The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. For optoporation of the opsin plasmids, the cells were plated on poly-D-lysine-coated glass-bottomed 35 mm Petri dishes (MatTek Corporation, MA, USA). Visualization of the reporter (YFP or Citrine) fluorescence under suitable illumination allowed the identification of transgene-expressing cells. The cells were incubated with all-trans-retinal (ATR, 1 μM) for at least 6 h before the patch-clamp experiments were performed.

Setup for optoporation

A schematic diagram of the experimental setup for optoporation is shown in Figure 1a. A beam (pulse width: ~ 100 fs) from a tunable Ti:sapphire laser (L1, Newport Spectra-Physics, Inc., Santa Clara, CA, USA) was expanded (by a beam expander, BE) and directed toward the sample by a dichroic mirror (DM1) through an inverted microscope (Nikon, Ti:U Eclipse). A $100\times$ (numerical aperture (NA) = 1.4) microscope objective was used to focus the laser beam to a diffraction-limited spot (Figure 1b) on the top surface of the cell. The focal plane of the laser was adjusted to match the imaging plane. Excitation (Ex) and emission (Em) filters were used to transmit and collect the appropriate bands of visible light to and from the sample and to block any remaining backscattered laser light. A second dichroic mirror (DM2) was used to reflect the fluorescence excitation light from the mercury lamp (FL). The images were acquired by a cooled EMCCD (Cascade 1K, Photometrics, Tucson, AZ, USA) and processed using ImageJ software (NIH). The number of fs laser pulses irradiating each cell was controlled by an external electromechanical shutter (S, Uniblitz, Vincent Associates, NY, USA). The laser beam power at the sample site was controlled by the laser (Mai Tai) interface software through alterations to the orientation of the polarizer (P). The average beam power at the sample site was calculated by multiplying the transmission factor of the microscope objective²⁷ by the average power

Figure 1 Targeted ultrafast laser-assisted opsin delivery setup and beam parameters. **(a)** Optoporation setup. L1, Ti:sapphire laser; BE, beam expander; S1–S3, shutters; P, polarizer; FL, fluorescence excitation; Ex/Em, excitation/emission filters; MO, microscope objective; WL, white-light source; CL, condenser lens; DM1 and DM2, dichroic mirrors; M, mirror. **(b)** Laser spot measured at the focal plane. Scale bar: 10 μm . **(c)** Intensity distribution in the XZ plane in the focal volume (wavelength: 850 nm, focused by an objective with NA = 1.4). **(d)** Schematic illustration of the optical delivery of opsin-encoding plasmids to target cells (lightning symbol) leading to selective opsin expression (detected by a fluorescent reporter protein).

measured at the back aperture of the objective. The laser parameters (energy per pulse and exposure time) were optimized (pulse energy: 1 nJ, exposure time: 20 ms, repetition rate: 76 MHz) and held constant in all experiments. Gridded glass-bottomed Petri dishes (MatTek, Ashland, MA, USA) were used to locate the exact positions of optoporation. In certain cases, when the cells were in a monolayer and the grid could not be visualized, a permanent marking pen with a sharp tip was used to mark small circles (radius: ~ 0.3 mm) on the bottom of the glass Petri dish, and a diamond marker was used to mark a cross within each circle.

Opsin expression and optogenetic stimulation

The expression of the opsins was observed after 36 h of transfection (lipofection or optoporation) via epifluorescence microscopy of the reporter proteins using a $20\times$ or $100\times$ microscope objective. The one-tailed *t*-test was used to determine the statistical significance of the difference in fluorescence expression. Broadband white light from a halogen lamp was coupled to a multimode optical fiber for optogenetic stimulation. A power meter (PM 100D, Thorlabs, Inc., NJ, USA)

was used to measure the light power at the sample plane. An electro-mechanical shutter was used to control the duration of the stimulation pulses. The shutter was synchronized with the patch-clamp recording system (Molecular Devices, Sunnyvale, CA, USA).

Opto-electrophysiology setup

The opto-electrophysiology setup consisted of an upright fluorescence microscope (Olympus, BX 60M) and an amplifier system (Axon Multiclamp 700B, Molecular Devices). For whole-cell patch-clamp experiments, borosilicate micropipettes with resistances of 3–4 M Ω were prepared using a pipette puller (Sutter Instruments, Novato, CA, USA). Each micropipette was filled with a solution of 130 mM K-gluconate, 7 mM KCl, 2 mM NaCl, 1 mM MgCl₂, 0.4 mM EGTA, 10 mM HEPES, 2 mM ATP-Mg, 0.3 mM GTP-Tris, and 20 mM sucrose. The electrode was mounted on a motorized micromanipulator (Sutter Instruments, MP225). The standard extracellular solution of 150 mM NaCl, 10 mM glucose, 5 mM KCl, 2 mM CaCl₂, and 1 mM MgCl₂ was buffered with 10 mM HEPES (pH 7.3). The patched cells were maintained at -70

mV, and the output from the amplifier was digitized using a Digidata 1440 instrument (Molecular Devices). For the patch-clamp measurements, the hardware was interfaced with patch-clamp software (pClamp 10, Molecular Devices). For activation of the opsin-expressing cells, the stimulation beam was delivered by a liquid light guide. The electromechanical shutter in the light path was interfaced with a PC for generation and control of the light pulses for optogenetic stimulation. The patch-clamp recording system was electrically isolated using a Faraday cage. pClamp 10 software (Molecular Devices) was used for patch-clamp data analysis.

RESULTS AND DISCUSSION

Transfection of a single opsin and multiple opsins via lipofection

To evaluate the transfection of mammalian cells with plasmids encoding either a single opsin or multiple opsins via lipofection, the molecular engineering of single and multiple opsins (a fusion of ChR2, C1V1, and ReaChR named 'White-opsin') was performed. Supplementary Fig. S1 shows vector maps of ChR2 (a), ReaChR (b), and White-opsin (c). The corresponding gel electrophoresis images of each opsin are shown in Supplementary Fig. S1d. HEK293 cells were transfected using Lipofectamine and plasmids at a final concentration of $1 \mu\text{g mL}^{-1}$, as measured by the absorption spectra of cloned ChR2, ReaChR, and White-opsin (Supplementary Fig. S1e–S1g). The quantification results for the Lipofectamine-based transfection of the different opsins are shown in Figure 2. In Figure 2a and 2b, we show the representative bright-field and fluorescence images of HEK293 cells transfected with ChR2-YFP using Lipofectamine. To quantify expression in the plasma membrane and the intracellular components, lines were drawn across representative cells, and the fluorescence intensity profiles of the opsin-reporter protein (YFP) along these lines were plotted (Figure 2c). A quantitative comparison of the expression of ChR2 in the membrane and intracellular components is shown in Figure 2d. Similarly, the expression levels of ReaChR (Figure 2e–2h) and White-opsin (Figure 2i–2l) after transfection via lipofection were quantified. Regardless of the type of opsin, the cellular morphology was observed to be compromised, changing from a flat to a spherical shape. This resulted in higher intracellular fluorescence, leading to the absence of any distinct peaks (across the membrane), as seen in Figure 2c, 2g, and 2k. Therefore, no significant differences between membrane and intracellular expression were observed (Figure 2d, 2h, and 2l).

Targeted laser-assisted transfection of single opsins

To achieve the targeted transfection of cells with opsin-encoding plasmids (ChR2 and ReaChR), we used optimized laser parameters²³ for optoporation into HEK293 cells. Before optoporation, the extracellular medium was exchanged with new DMEM medium-containing DNA plasmids (ReaChR or ChR2) with a final concentration of $1 \mu\text{g mL}^{-1}$ and incubated for 30 min. Figure 1a shows the setup for targeted ultrafast laser-assisted opsin delivery. The focused laser spot is shown in Figure 1b; the simulated intensity distribution of the spot in the XZ plane in the focal volume (wavelength: 850 nm, focused by an objective with NA = 1.4) is presented as Figure 1c. Using this localized focal volume, spatially targeted cells can be selectively optoporated, as schematically illustrated in Figure 1d. Figure 3 shows the targeted fs laser-assisted transfection of single opsins into HEK293 cells. A representative fluorescence image of targeted ChR2-YFP-optoporated HEK293 cells (Figure 3a) is overlaid on the corresponding bright-field image (Figure 3b). Similarly, Figure 3c and 3d shows representative laser-targeted ReaChR-transfected HEK293 cells. Unlike in the case of

Lipofectamine-based transfection, the cellular morphology after laser transfection was found to remain flat. The fluorescence intensity profiles of the opsin-reporter proteins (YFP and Citrine) along the lines drawn across representative cells (Figure 3e and 3f) exhibit distinct peaks at the plasma membranes, implying the localized expression of ChR2 and ReaChR on the plasma membrane (Figure 3g and 3h). Figure 4e shows a quantitative comparison between the ChR2 and ReaChR expression distributions (ratio of membrane to intracellular expression) after optoporation and lipofection.

Laser-assisted transfection of White-opsin: functional evaluation using broadband light

Figure 4 shows the targeted laser-assisted transfection of HEK293 cells with White-opsin (fusion of multiple opsins) and the subsequent broadband (white)-light-based optogenetic stimulation. Visualizations of the transfection of HEK293 cells with White-opsin plasmids via optoporation or lipofection are shown in Supplementary Fig. S2. An increase in intracellular fluorescence and a few distinct specks (attributed to the propidium iodide staining of White-opsin plasmids) after optoporation (at the site marked by an arrow in Supplementary Fig. S2a) were observed 1 min after optoporation (Supplementary Fig. S2c). The transfected plasmid(s) were transcribed and translated into proteins (i.e., White-opsin and the reporter YFP), which were expressed in the targeted organelle (i.e., the plasma membrane). Similar to the ChR2- and ReaChR-optoporated cells, the White-opsin delivered by the fs laser was found to be preferentially expressed on the plasma membrane (Figure 4a). The non-optoporated (control) cells were observed not to express the fluorescent reporter protein. Figure 4b shows the representative fluorescence intensity profiles of the reporter proteins along lines drawn across cells (e.g., the dotted white line in Figure 4a). A quantitative comparison of the expression in the membranes and intracellular components of cells optoporated with White-opsin is shown in Figure 4c. To evaluate the functional activation of White-opsin, broadband stimulation was applied to the White-opsin-expressing cells and patch-clamp electrophysiology was used to record the photoinduced currents. Representative inward photocurrents generated in response to white-light optogenetic stimulation (0.12 mW mm^{-2}) at two different pulse widths are shown in Figure 4d; these results suggest that low-intensity broadband light can be used to generate a robust photocurrent in a spatially targeted manner with high temporal resolution. The white-light-induced photocurrent generated in the White-opsin-expressing cells (Figure 4d) was significantly higher than the photocurrent in cells expressing ChR2 (Supplementary Fig. S3).

Quantitative comparison between two non-viral gene delivery methods

Calculation of the total intensities from individual cells revealed no significant differences between lipofection and optoporation. Figure 4e summarizes the quantitative comparison between the different opsin expression distributions (ratios of membrane to intracellular expression) resulting from the two different non-viral gene delivery methods. The higher membrane-to-intracellular ratio of expression in optoporated cells than lipofected cells (Figure 4e) can be attributed to the flat morphology of the optoporated opsin-expressing cells compared with those transfected via lipofection, which tended to become spherical. This alteration in the morphology of lipofected cells was confirmed by three-dimensional confocal microscopy (Supplementary Fig. S4).

Figure 2 Quantification results for the Lipofectamine-based transfection of different opsins. (a–d) ChR2-, (e–h) ReaChR-, and (i–l) White-opsin-transfected HEK293 cells. (a, e, i) Bright-field and (b, f, j) fluorescence images of HEK293 cells transfected with ChR2-YFP, ReaChR, and White-opsin, respectively, imaged using a 20 $\times$ objective. Scale bar: 20 μm . (c, g, k) Representative intensity profiles of the opsin-reporter protein fluorescence along the lines drawn across the cells. (d, h, l) Quantitative comparison of expression in membrane and intracellular components in ChR2-, ReaChR-, and White-opsin-transfected cells. $N = 6/\text{opsin}$. Average $\pm$ S.D. No significant difference is observed between the membrane and the intracellular components. Flour., fluorescence.

Although the use of opsins to modulate cellular activity has been rapidly increasing, the existing methods (e.g., virus-mediated approaches and lipofection) of delivering those opsins lack the ability to achieve targeted expression. Traditional methods of targeted delivery of impermeable molecules into cells require microinjection using a mechanical needle or microelectrodes, which often causes cell death and compromises sterility. Carbon^{28,29} and gold^{30,31} nanoparticles in conjunction with pulsed²⁸ or continuous-wave²⁹ laser beams have been employed for the delivery of exogenous small molecules to cells; this approach requires the presence of the nano-

particles along with the molecules to be delivered in the vicinity of the membrane of the target cell. Furthermore, in viral delivery methods, the size of the plasmids that can be packaged for delivery is limited, which may restrict the use of these methods to the delivery of only single-opsin plasmids rather than multiple opsins (such as White-opsin). The optoporation of one or more opsin plasmids into cells offers significant advantages over other methods of delivery (e.g., micro-injection, electroporation, lipofection, and viral transfection) because this method provides the ability to deliver large therapeutic molecules (e.g., >10 kb), spatial and temporal

Figure 3 Targeted laser-assisted transfection of single opsins. **(a)** Representative fluorescence image of targeted ChR2-YFP-optoporation HEK293 cells, **(b)** overlaid on the corresponding bright-field image showing highly packed cells in a monolayer, imaged using a 20× objective. **(c)** Representative fluorescence image of targeted HEK293 cells optoporation with ReaChR, **(d)** overlaid on the corresponding bright-field image showing cells in a monolayer, imaged using a 100× objective. Scale bar: 15 μm. **(e, f)** Representative fluorescence intensity profiles of the reporter proteins along the lines drawn across the cells (white lines in **a, c**). **(g, h)** Quantitative comparison of expression in the membrane and the intracellular components in cells optoporation with ChR2 and ReaChR. $N = 5/\text{opsin}$. Average $\pm$ S.D. * $p < 0.01$ difference between the membranes and intracellular components of opsin-expressing cells. Fluor., fluorescence.

selectivity, and absolute sterility. Furthermore, it can be used for the delivery of multiple-opsin plasmids into a single cell or for the delivery of single-opsin plasmids into multiple cells through multiplexing of the laser beam.

A substantial barrier to the clinical translation of optogenetic methods of vision restoration is the delivery of opsin-encoding genes into spatially targeted regions of a retina that has degenerated. For example, the spatially targeted delivery of opsin-encoding genes to either the peripheral retina or macula, which will begin to lose light sensitivity through the loss of photoreceptors, is required in cases of RP and AMD, respectively. Furthermore, patient-to-patient variability and time-dependent changes in the spatial distribution of retinal degeneration demand the site-specific expression of opsins. Unlike viral methods, the use of spatially targeted optoporation will enable the

expression of one or more opsins in areas of retinal degeneration, thereby allowing for effective vision restoration. Using viral or other non-viral (e.g., electroporation and lipofection) methods, the constructs will be delivered universally, inducing uncontrolled expression over the entire retina and perturbing the already functioning retinal regions. Furthermore, the delivery of macro-constructs encoding multiple opsins with spectrally separated activation peaks over the entire visible spectrum will lead to higher photocurrents in response to ambient white light. This will enable the generation of action potentials in White-opsin-sensitized higher-order neurons at a much lower spectral density of intensity (intensity/bandwidth) than can be achieved through the conventional use of monochromatic light and single narrow-band opsins. Although different viral vectors can separately deliver different plasmids encoding for opsins for the

Figure 4 Targeted laser-assisted transfection of multiple opsins: functional evaluation and quantitative comparison between two non-viral delivery methods. **(a)** Fluorescence image of HEK293 cells after optoporation with White-opsin, imaged using a 100× objective. Scale bar: 15 μ m. **(b)** Representative fluorescence intensity profiles of reporter proteins along lines drawn across cells (e.g., the dotted white line in **a**). **(c)** Quantitative comparison of the expression in the membranes and intracellular components of cells optoporated with White-opsin. $N = 5$. Average $\pm$ S.D. * $p < 0.01$ difference between the membranes and intracellular components of White-opsin-expressing cells. **(d)** Representative inward photocurrent profiles in response to white-light optogenetic stimulation (0.12 mW mm^{-2}) at two different pulse widths (100 and 200 ms). **(e)** Quantitative comparison between the opsin expression distributions (ratios of membrane to intracellular expression) observed after optoporation and lipofection. $N = 7$ /opsin/delivery method. Average $\pm$ S.D. * $p < 0.05$ difference between membrane and intracellular components. Flour., fluorescence.

different spectral components, identical stoichiometric expressions of the different spectrally sensitive components in each cell cannot be guaranteed. Therefore, we believe that the application of the spatially targeted laser transfection of macro-constructs such as White-opsin into a retina that has degenerated will allow the efficient stimulation of higher-order retinal cells in areas of photoreceptor degeneration with ambient white light, leading to visual restoration.

The surgical risks of existing complex retinal-implantation procedures for improving vision are very high. This is a particular concern when an implant must be replaced because of device failure during chronic usage. Our method of laser-based targeted delivery of opsins is easy to incorporate into current clinical practice via the intravitreal injection of multiple-opsin-encoding plasmids followed by the ultrafast NIR laser-based non-contact creation of highly localized, transient

pores on the outer RGC membrane in photodegenerated areas. A reasonable delivery time of ~ 45 min for an area containing 500 000 cells is expected for 5 ms of exposure per cell. Furthermore, in the case of loss of opsin expression or the degeneration of new retinal area(s), the entire process can be easily repeated. For *in vitro* or small animal models, this method can be practiced using any commercial multiphoton microscope, and the same system can be used to both transfect and stimulate (via two-photon stimulation¹⁰) opsin-sensitized cells with high spatial accuracy.

CONCLUSIONS

To conclude, an ultrafast NIR laser microbeam was used to deliver both single opsins and large multi-opsin constructs to target cells. The effectiveness and function of the multiple-opsin-sensitized cells were

confirmed via patch-clamp electrophysiology. The NIR optoporation method will eliminate the requirement for multiple virus delivery and the possible immunogenic reactions, as well as offering the ability to deliver macro-constructs encoding opsins that are sensitive to a broad spectral range to spatially targeted areas of retinal degeneration. Such spatially resolved delivery of genes to the peripheral retina/macula is ideal for sensitizing retinal areas that have degenerated, thus paving the way toward restoring lost vision in RP/AMD patients. This will allow a higher sensitivity of opsin-sensitized higher-order neurons in targeted retinal areas (those suffering from photodegeneration) to ambient white light and, therefore, will significantly lower the activation threshold compared with the conventional approaches based on narrow-band opsins and/or active stimulation based on intense narrow-band light.

CONFLICT OF INTEREST

Dr. Samarendra Mohanty is a founder of, and has an equity interest in NanoScope Technologies, LLC, which is developing products in Biomedical diagnostics and therapeutic technologies. The terms of this arrangement have been reviewed and approved by the University of Texas at Arlington in accordance with its policy on objectivity in research.

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